厚生労働科学研究費補助金（政策創薬総合研究推進事業）
研究分担報告書

婦人科腫瘍、胎児組織の移植維持系の確立

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研究要旨：婦人科癌における腫瘍増殖のメカニズムの解明に向けて上皮―間質相互関与を解析する移植モデルを確立した。その研究システムにおいて腫瘍間質が HB-EGF 蛋白をオートクリンあるいはパラクリン的に分泌し、子宮頸癌進展に重要な役割を持つことを示した。

A. 研究目的

抗癌剤耐性のメカニズムを解明するための Key として上皮―間質相互作用に着目した。上皮、間質を個別あるいはともに培養する婦人科癌の移植系を確立し、上皮―間質相互作用関わる分子を同定することを目的とした。

B. 研究方法

1．子宮頸癌組織のうち上皮成分と間質を個々に培養し、移植モデルを確立し上皮―間質相互作用が腫瘍増殖に与える影響を検討した。

2. HB-EGF に注目し、子宮頸癌細胞株を用いてノックダウンあるいは過剰発現させることにより腫瘍の増殖能の違いを解析する。

3. 手術摘出標本のパラフィン包埋切片を HB-EGF 抗体を用いて免疫染色を行い発現の有無・程度を解析する。

（倫理面への配慮）
研究に際し、細胞株以外に臨床検体の採取・利用も不可欠であるが、インフォームドコンセントを得たうえで、手術で摘出された組織を用いるため、患者に危害が及ぶことはなかった。

C. 研究結果

1. in vitro 実験系およびマウスへの Xenograft 移植モデル実験より、子宮頸癌において上皮―間質共培養した腫瘍は、上皮単独培養の腫瘍よりも有意に増殖能が有意に高いことが明らかになった。そのメカニズムのひとつとして HB-EGF のオートクリン機構が重要であることを証明した。実際に子宮頸癌組織の免疫染色の結果からその間質に高発現することが証明された。(Murata T. et al, Cancer Res. 2011 Nov 1;71(21):6633-42. Epub 2011 Oct 18)。

D. 考察

上皮―間質相互作用は子宮頸癌の発生・進展に重要な役割を果たしており、治療の標的になりうると考えられる。HB-EGF はその候補分子のひとつであることが考えられる。

E. 結論

上皮―間質相互関与を破壊することが腫瘍の進展を阻止する可能性がある。これらの先駆的情報は今後、実際の臨床への応用の基盤となるものである。

F. 健康危険情報

G. 研究発表

1．論文発表

1) Mabuchi S, Okazawa M, Isohashi F, Matsuo K,Ohta Y, Suzuki O, Yoshioka Y, Enomoto T, Kamiura S, Kimura T. Radical hysterectomy with adjuvant radiotherapy versus definitive radiotherapy alone for FIGO stage IIB cervical cancer. Gynecol Oncol. 123(2):241-7, 2011.

2) Ueda Y, Okazawa M, Enomoto T, Egawa-TakataT, Kimura T, Yoshino K, Fujita M,Ohta Y, Kamiura S, Kimura T. Dissection of unsuspicious para-aortic lymph nodes does not improve prognosis of advanced endometrial carcinoma with intra- or extra-abdominal metastasis. Anticancer Res. (12):4513-7, 2011.

3) Murata T, Mizushima H, Chinen I, Moribe H, Yagi S, Hoffman RM, Kimura T,Yoshino K, Ueda Y, Enomoto T, Mekada E. HB-EGF and PDGF mediate reciprocal interactions of carcinoma cells with cancer-associated fibroblasts to supportprogression of uterine cervical cancers. Cancer Res. 71(21):6633-42, 2011.

4) Mabuchi S, Okazawa M, Isohashi F, Ohta Y, Maruoka S, Yoshioka Y, Enomoto T, Morishige K,Kamiura S, Kimura T. Postoperative whole pelvic radiotherapy plus concurrent chemotherapy versus

extended-field irradiation for early-stage cervical cancer patients with multiple pelvic lymph node metastases. Gynecol Oncol. 20(1):94-100, 2011.

5) Ugaki H, Kimura T, Miyatake T, Ueda Y, Yoshino K, Matsuzaki S, Fujita M,Kimura T, Morii E, Enomoto T. Intraoperative frozen section assessment of myometrial invasion and histology of endometrial cancer using the revised FIGO staging system. Int J Gynecol Cancer. 21(7):1180-4, 2011.

6) Matsuo K, Nishimura M, Bottsford-Miller JN, Huang J, Komurov K, Armaiz-Pena GN, Shahzad MM, Stone RL, Roh JW, Sanguino AM, Lu C, Im DD, Rosenshien NB,Sakakibara A, Nagano T,Yamasaki M, Enomoto T, Kimura T, Ram PT, Schmeler KM,Gallick GE, Wong KK, FrumovitzM, Sood AK. Targeting SRC in mucinous ovarian carcinoma. Clin Cancer Res. 17(16):5367-78, 2011.

7) Enomoto T, Yoshino K, Fujita M, Miyoshi Y, Ueda Y, Koyama S, Kimura T,Tomimatsu T, Kimura T. A successful case of abdominal radical trachelectomy for cervical cancer during pregnancy. Eur J Obstet Gynecol Reprod Biol. 158(2):365-6, 2011.

8) Egawa-Takata T, Ueda Y, Kuragaki C, Miyake T, Miyatake T, Fujita M, Yoshino K, Nakashima R, Okazawa M, Tsutsui T, Morishige K, Kimura T, Yamasaki M,Nishizaki T, Nagamatsu M, Ito K, Asada M, Ogita K, Wakimoto A, Yamamoto T, Nishio Y, Enomoto T. Chemotherapy forendometrial carcinoma (GOGO-EM1 study): TEC (paclitaxel, epirubicin, and carboplatin) is an effective remission-induction and adjuvant therapy. Cancer Chemother Pharmacol. 68(6):1603-10, 2011.

9) Miyake T, Ueda Y, Egawa-Takata T, Matsuzaki S, Yokoyama T, Miyoshi Y, Kimura T, YoshinoK, Fujita M, Yamasaki M, Enomoto T, Kimura T. Recurrent endometrial carcinoma: prognosis for patients with recurrence within 6 to 12 monthsis worse relative to those relapsing at 12 monthsor later. Am J Obstet Gynecol. 204(6):535.e1-5, 2011.

10) Ueda Y, Miyake T, Egawa-Takata T, Miyatake T, Matsuzaki S, Yokoyama T,Yoshino K, Fujita M, Enomoto T, Kimura T. Second-line chemotherapy for advanced or recurrent endometrial carcinoma previously treated with paclitaxel and carboplatin, with or without epirubicin. Cancer Chemother Pharmacol. 67(4):829-35, 2011.

11) Rahadiani N, Ikeda J, Mamat S, Matsuzaki S, Ueda Y, Umehara R, Tian T, Wang Y, Enomoto T, Kimura T, Aozasa K, Morii E. Expression ofaldehyde dehydrogenase 1 (ALDH1) in endometrioid adenocarcinoma and its clinical implications.
Cancer Sci. 102(4):903-8, 2011.

12) Fujwara K, Yoshino K, Enomoto T, Fujita M, Ueda Y, Miyatake T, Kimura T,Muraji M, Fujita H, Kimura T, Hori M. Usefulness of computed tomography in predicting cytoreductive surgicaloutcomes for ovarian cancer. Arch Gynecol Obstet. 284(6):1501-7, 2011.

13) Yoshino K, Enomoto T, Fujita M, Ueda Y, Kimura T, Kobayashi E, Tsutsui T,Kimura T. Salvage chemotherapy for recurrent or persistent clear cell carcinoma of the ovary: a single-institution experience for a series of 20 patients. Int JClin Oncol. 2011 Dec 10. [Epub ahead of print]PubMed PMID: 22160560.

14) Fujiwara K, Egawa-Takata T, Ueda Y, Kimura T, Yoshino K, Fujita M, Miyatake T,Ohta Y, Kamiura S, Enomoto T, Kimura T. Investigating the relative efficacies of combination chemotherapy of paclitaxel/carboplatin, with or without anthracycline, for endometrial carcinoma. Arch Gynecol Obstet. 2011 Nov 30. [Epub ahead of print] PubMed PMID: 22127553.

15) Miyoshi Y, Miyatake T, Ueda Y, Morimoto A, Yokoyama T, Matsuzaki S, Kimura T, Yoshino K, Fujita M, Ohashi H, Morii E, Enomoto T, Kimura T. Prediction, based on resection margins,of long-term outcome of cervical intraepithelial neoplasia 3 treated by Shimodaira-Taniguchi conization. Arch Gynecol Obstet. 2011 Nov 23. [Epub ahead of print] PubMed PMID: 22109382.

16) Ueda Y, Enomoto T, Kimura T, Yoshino K, Fujita M, Kimura T. Two distinct pathways to development of squamous cell carcinoma of the vulva. J Skin Cancer.2011;2011:951250. Epub 2010 Sep 28. PubMed PMID: 21188235; PubMed Central PMCID: PMC3003991.

2. 学会発表

H. 知的財産権の出願・登録状況
（予定を含む。）

1. 特許取得 なし
2. 実用新案登録 なし
3. その他 特になし

III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Masao Iwamori, Akihiro Sakai, Norihito Minamimoto, Yuriko Iwamori, Kyoko Tanaka, Daisuke Aoki, Shigeki Adachi and Taisei Nomura	Characterization of novel glycolipid antigens with an β-galactose epitope in lactobacilli detected with rabbit anti-Lactobacillus antisera and occurrence of antibodies against them in human sera.	J. Biochem.	150	515-523	2011
Miyake T, Ueda Y, Matsuzaki S, Miyatake T, Yoshino K, Fujita M, Nomura T, Enomoto T, Kimura T.	CRABP1-reduced Expression is associated with poorer prognosis in serous and clear cell ovarian adenocarcinoma.	J Cancer Res Clin Oncol.	13	715-722	2011
Dillip Kumar Parida1, Koji Wakame, Taisei Nomura	Integrating Complimentary and Alternative Medicine in Formof Active Hexose Co-Related Compound (AHCC) in the Management of Head & Neck Cancer Patient.	International Journal of Clinical Medicine	2	588-592	2012
野村大成、足立成基、梁 治子、畑中英子、菊谷 理絵、時田偉子、堀家なな緒、中島裕夫、本行忠志、藤川和男、伊藤哲夫、落合俊昌、行徳淳一郎、若命浩二、D. K. Parida、R. I. Bersimbay	宇宙環境の人体影響評価と防護に関する研究；放射線晩発影響の防護	Space Util. Res.	28		2012（3月、in press）
Saito S, Morita K, Kohara A, Masui T, Sasao M, Ohgushi H, Hirano T.	Use of BAC array CGH for evaluation of chromosomal stability of clinically used human mesenchymal stem cells and of cancer cell lines.	Hum Cell. :(2011)	24(1)	2-8	2011

Mimura S, Kimura N, Hirata M, Tateyama D, Hayashida M, Umezawa A, Kohara A, Nikawa H, Okamoto T, Furue MK.	Growth factor-defined culture medium for human mesenchymal stem cells.	Int J Dev Biol.	55(2)	181-187	2011
菅三佳，高田圭，小原有弘，末盛博文，青井貴之，中村幸夫，古江- 楠田美保	ヒト多能性幹細胞の命名法の国際統一規格案について.	再生医療	vol.11	2-8	2011
Hatano K, Miyamoto Y, Nonomura N, Kaneda Y.	Expression of gangliosides, GD1a and sialyl paragloboside, is regulated by NF-κB-dependent transcriptional control of α2,3-sialyltransferase I, II and VI in human castration-resistant prostate cancer cells.	Int J Cancer	129	1837-1847	2011
Hatano K, Nonomura N, et al.	Retrospective analysis of an oral combination of dexamethasone, uracil plus tegafur and cyclophosphamide for hormone-refractory prostate cancer	Jpn J Clin Oncol	41	253-259	2011
Nonomura N, Takayama H et al.	Infiltration of tumour-associated macrophages in prostate biopsy specimens is predictive of disease progression after hormonal therapy for prostate cancer.	BJU Int	107	1918-1922	2011
S. Mabuchi, M. Okazawa, F. Isohashi, Y. Ohta, S Maruoka, Y. Yoshioka, T. Enomoto, K. Morishige, S. Kamiura, T. Kimura	Postoperative whole pelvic radiotherapy plus concurrent chemotherapy versus extended-field irradiation for early-stage cervical cancer patients with multiple pelvic lymph node metastases.	Gynecol. Oncol.	120	94-100	2011

Ueda Y, Okazawa M, Enomoto T, Egawa-Takata T, Kimura T, Yoshino K, Fujita M,Ohta Y, Kamiura S, Kimura T.	Dissection of unsuspicious para-aortic lymph nodes does not improve prognosis of advanced endometrial carcinoma with intra- or extra-abdominal metastasis.	Anticancer Res.	31(12)	4513-8	2011
Murata T, Mizushima H, Chinen I, Moribe H, Yagi S, Hoffman RM, Kimura T, Yoshino K, Ueda Y, Enomoto T, Mekada E.	HB-EGF and PDGF mediate reciprocal interactions of carcinoma cells with cancer-associated fibroblasts to support progression of uterine cervical cancers.	Cancer Res.	71(21)	6633-42	2011
Mabuchi S, Okazawa M, Isohashi F, Matsuo K, Ohta Y, Suzuki O, Yoshioka Y, Enomoto T, Kamiura S, Kimura T.	Radical hysterectomy with adjuvant radiotherapy versus definitive radiotherapy alone for FIGO stage II B cervical cancer.	Gynecol Oncol.	123(2)	241-7	2011
Ugaki H, Kimura T, Miyatake T, Ueda Y, Yoshino K, Matsuzaki S, Fujita M, Kimura T, Morii E, Enomoto T.	Intraoperative frozen section assessment of myometrial invasion and histology of endometrial cancer using the revised FIGO staging system.	Int J Gynecol Cancer.	21(7)	1180-4	2011
Matsuo K, Nishimura M, Bottsford-Miller JN, Huang J, Komurov K, Armaiz-Pena GN, Shahzad MM, Stone RL, Roh JW, Sanguino AM, Lu C, Im DD, Rosenshien NB,Sakakibara A, Nagano T, Yamasaki M, Enomoto T, Kimura T, Ram PT, Schmeler K M,Gallick GE, Wong KK, Frumovitz M, Sood AK.	Targeting SRC in mucinous ovarian carcinoma.	Clin Cancer Res.	17(16)	5367-78	2011

Enomoto T, Yoshino K, Fujita M, Miyoshi Y, Ueda Y, Koyama S, Kimura T, Tomimatsu T, Kimura T.	A successful case of abdominal radical trachelectomy for cervical cancer during pregnancy.	Eur J Obstet Gynecol Reprod Biol.	158(2)	365-6	2011
Egawa-Takata T, Ueda Y, KuragakiC, Miyake T, Miyatake T, Fujita M, Yoshino K, Nakashima R, Okazawa M, Tsutsui T, Morishige K, Kimura T, Yamasaki M, Nishizaki T, Nagamatsu M, Ito K, Asada M, Ogita K, Wakimoto A, Yamamoto T, Nishio Y, Enomoto T.	Chemotherapy for endometrial carcinoma (GOGO-EM1 study): TEC (paclitaxel, epirubicin, and carboplatin) is an effective remission-induction and adjuvant therapy.	Cancer Chemother Pharmacol.	68(6)	1603-10	2011
Miyake T, Ueda Y, Egawa-Takata T, Matsuzaki S, Yokoyama T, Miyoshi Y, Kimura T, Yoshi no K, Fujita M, Yamasaki M, Enomoto T, Kimura T.	Recurrent endometrial carcinoma: prognosis for patients with recurrence within 6 to 12 months is worse relative to those relapsing at 12 months or later.	Am J Obstet Gynecol	204(6)	535.e1-5	2011
Ueda Y, Miyake T, Egawa-Takata T, Miyatake T, Matsuzaki S, Yokoyama T, Yoshino K, Fujita M, Enomoto T, Kimura T.	Second-line chemotherapy for advanced or recurrent endometrial carcinoma previously treated with paclitaxel and carboplatin, with or without epirubicin.	Cancer Chemother Pharmacol.	67(4)	829-35	2011

N. Rahadiani, J. Ikeda J, S. Mamat, S. Matsuzaki, Y. Ueda, R. Umehara, Tian T, Wang Y, T. Enomoto, T. Kimura, K. Aozasa, E. Morii	Expression of aldehyde dehydrogenase 1 (ALDH1) in endometrioid adenocarcinoma and its clinical implications.	Cancer Sci.	102(4)	903-8	2011
K. Fujwara, K. Yoshino, T. Enomoto, M. Fujita, Y. Ueda, T. Miyatake, T. Kimura, M. Muraji, H. Fujita, T. Kimura, M. Hori.	Usefulness of computed tomography in predicting cytoreductive surgical outcomes for ovarian cancer.	Arch. Gynecol. Obstet.	284(6)	1501-7	2011
Yoshino K, Enomoto T, Fujita M, Ueda Y, Kimura T, Kobayashi E, TsutsuiT, Kimura T.	Salvage chemotherapy for recurrent or persistent clear cell carcinoma of the ovary: a single-institution experience for a series of 20patients.	Int J Clin Oncol.			2011 in press
Fujiwara K, Egawa-Takata T, Ueda Y, Kimura T, Yoshino K, Fujita M, Miyatake T, Ohta Y, Kamiura S, Enomoto T, Kimura T.	Investigating the relative efficacies of combination chemotherapy of paclitaxel/ carboplatin, with or without anthracycline, for endometrial carcinoma.	Arch Gynecol Obs tet.	285	1447-1453	2012
Miyoshi Y, Miyatake T, Ueda Y, Morimoto A, Yokoyama T, Matsuzaki S, Kimura T, Yoshino K, FujitaM, Ohashi H, Morii E, Enomoto T, Kimura T.	Prediction, based on resection margins, of long-term outcome of cervical intraepithelial neoplasia 3 treated by Shimodaira-Taniguchi conization.	Arch Gynecol Obstet.	285	1427-1432	2012

Y. Ueda, T. Enomoto, T. Kimura, K. Yoshino, M. Fujita, T. Kimura	Two distinct pathways to development of squamous cell carcinoma of the vulva.	J. Skin Cancer			2011 in press

Ⅳ．研究成果の刊行物・別刷

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Characterization of novel glycolipid antigens with an α-galactose epitope in lactobacilli detected with rabbit anti-*Lactobacillus* antisera and occurrence of antibodies against them in human sera

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Anti-*Lactobacillus johnsonii* (LJ) antisera generated by immunization of rabbits with LJ reacted with glycero-glycolipids in LJ, i.e. dihexaosyl diacylglycerol (DH-DG), trihexaosyl DG (TH-DG) and tetrahexaosyl DG (TetH-DG), whose reactivities with antisera increased proportionally with longer carbohydrate chains of glycolipids. Structural analyses of glycolipids from LJ revealed that DH-DG was Galα1-2Glcα1-3′DG, and TH-DG and TetH-DG were novel derivatives of it with α-Gal at the non-reducing terminal, i.e. Galα1-6Galα1-2Glcα1-3′DG and Galα1-6Galα1-6Galα1-2Glcα1-3′DG, respectively. DH-DG was commonly present in several lactobacilli examined, but TetH-DG was restricted to LJ, *L. intestinalis* and *L. reuteri*, while the TH-DGs from *L. casei* were Glc1-6Galα1-2Glcα1-3′DG and an esterified derivative of it, Glc1-6Galα1-2Glc(6-fatty acid)α1-3′DG, as reported in the literature. Anti-LJ antisera reacted with TH-DG and esterified TH-DG from *L. casei* to lesser extents, but not at all with gentibiosyl DG from *Staphylococcus epidermidis* or kojibiosyl DG from *Streptococcus salivalis* or sphingoglycolipids containing α-Gal residues. The major molecular species of glycolipids obtained from lactobacilli were 11-octadecenoic and 11,12-methylene-octadecanoic acids-containing ones. Also, human IgM antibodies against TH-DG and TetH-DG from LJ were detected in human sera, with various antibody titres, indicating that an immune reaction to symbiotic lactobacilli occurs against their glycolipid antigens, TH-DG and TetH-DG.

Keywords: antigenic determinants/carbohydrate structure/glycoglycerolipids/Gram-positive bacteria/human antibodies.

Abbreviations: CL, cardiolipin; DG, diacyl glycerol; Gal, galactose; Glc, glucose; GalU, galacturonic acid; GlcU, glucuronic acid; Man, mannose; PG, phosphatidylglycerol; MH-DG, monohexaosyl diacylglycerols; DH-DG, dihexaosyl diacylglycerols; TH-DG, trihexaosyl diacylglycerols; TetH-DG, tetrahexaosyl diacylglycerols; TEA, triethanolamine; TMS, trimethylsilyl.

Gg_4Cer in the murine digestive tract has been revealed to be selectively distributed in the small intestine, where it provides a receptor structure for the formation of bacterial flora including symbiotic bacteria, e.g. *Lactobacillus johnsonii* (LJ), in the intestinal epithelia (*1*). During the course of exploration of the receptor function of epithelial glycolipids, we prepared anti-*Lactobacillus* antisera by immunization of rabbits with a murine intestinal *Lactobacillus*, LJ, and found that the antisera contained anti-Gg_4Cer antibodies due to the presence of a Gg_4Cer-mimicking structure in the protein, but not the lipid fraction of LJ, suggesting that an epitope structure common to both lactobacilli and the host is used for evading immunological surveillance by the host for symbiosis (*1*, *2*). The major antigens, other than the Gg_4-mimicking structure, recognized by anti-LJ antisera were glycolipids, which were present in equivalent amounts to those of phospholipids in LJ (*1*, *2*). In the case of Gram-positive bacteria, the cell wall is constructed from peptidoglycan and teichoic acids, which are essential for the bacteria to adapt to severe circumstances, including as to temperature, pH, ionic strength, superoxide, and attack by enzymes and immunocytes (*3*). Teichoic acids, whose major structure is poly-1,5-ribitol phosphate, poly-1,3-glycerol phosphate, poly-1,2-glycerolphosphate, or poly-trihexaosylphosphate, are anchored on glyceroglycolipids as lipoteichoic acid in the cytoplasmic membrane (*4*). For instance, the structures of lipoteichoic acid in the cell wall of *Streptococcus* species have been characterized as poly-1,3-glycerol phosphate-6Glcα1-2Glcα1-3′ diacylglycerol and poly(6Galα1-6Galα1-3″,Galα1-2″,glycerol-1″-phosphate)-6Glcα1-2Glc(6-fatty acid)α1-3′diacylglycerol (*5*, *6*). In general, dihexaosyl diacylglycerols (DH-DGs) are the major

lipid constituents of several Gram-positive bacteria, but their structures are bacterial species-characteristic, i.e. Glcα1-2Glcα1-3′DG for *Streptococcus* and Acholeplasma, Glcβ1-6Glcβ1-3′DG for *Staphylococcus* and Mycoplasma, Galβ1-2Galβ1-3′DG for Bifidobacterium, Galα1-2Glcα1-3′DG for *Lactobacillus*, Glcβ1-4GlcUα1-3′DG for Pseudomonas, Galα1-4GalUα1-3′DG for Streptomyces, Manα1-3Manα1-3′DG for Micrococcus, and Galβ1-6Galβ1-3′DG for Arthrobacter (*5*, *7*). Antibodies produced on immunization of rabbits with bacteria have been shown to frequently react with bacterial specific DH-DG, and has been applied as a probe to discriminate bacterial species for serological classification, indicating that glycolipids are exposed on the bacterial cell wall and function as bacterial antigens, probably for mutual recognition among bacteria and immune recognition by the host animals (*8*, *9*). In this connection, an immune reaction toward symbiotic or infecting bacteria in humans has been shown to contribute to the production of natural antibodies including anti-blood group ones in human sera, as well as to the onset of diseases, such as multiple sclerosis (*9*, *10*). The anti-LJ antisera, as described above, also reacted with DH-DG Galα1-2Glcα1-3′DG, as a *Lactobacillus*-characteristic glycolipid, in the lipid fraction of LJ, but additional glycolipids, which migrated to positions corresponding to tri- and tetrahexaosyl diacylglycerols (TH-DG and TetH-DG), were found to react more strongly than DH-DG (*1*, *2*). As to glycolipids from *Lactobacillus* species, Glcβ1-6Galα1-2Glcα1-3′DG and Glcβ1-6Glcβ1-6Galα1-2Glcα1-3′DG have been isolated from *L. casei*, as reported in the literature (*11*, *12*), but TH-DG and TetH-DG in LJ detected by antisera were found to be different from them and to have novel structures, Galα1-6Galα1-2Glα1-3′DG and Galα1-6Galα1-6Galα1-2Glcα1-3′DG, respectively. In addition, we examined whether or not antibodies toward them are generated in human sera as a result of immune reaction to symbiotic bacteria.

Materials and Methods

Bacteria

LJ (JCM No. 1022), *L. intestinalis* (JCM No. 7548), *L. reuteri* (JCM No. 1112), *L. casei* (JCM No. 1134), *L. fermentum* (JCM No. 1137), *L. plantarum* (JCM No. 1550), *L. rhamnosus* (JCM No.1561), *S. epidermidis* (JCM No. 2414), and *S. salivalis* (JCM No. 5707) were purchased from the Japan Collection of Microorganisms (JCM), RIKEN BioResource Center (Wako, Saitama, Japan). *S. aureus* (860) and *S. epidermidis* (866) and *Escherichia coli* (JM109) were obtained from the American Type Culture Collection (Rockville, MD, USA) and Invitrogen (Carlsbad, CA, USA), respectively. The culture media for bacteria were as follows: MRS broth (Beckton-Dickinson, Sparks, MD, USA) for lactobacilli, tryptic soy broth (Beckton-Dickinson) for *S. epidermidis*, heart infusion broth (Beckton-Dickinson) for *S. salivalis*, and LB broth (Nacalai Tesque, Kyoto) for *E. coli*.

Materials

Glycolipids, GlcCer, GalCer, LacCer, Gb_3Cer, Gb_4Cer, Gg_4Cer and $IV^2Fuc\alpha$-Gg_4Cer (*13*), were purified in our laboratory (*1*, *2*). Rabbit polyclonal antisera toward *LJ* (*1*) and murine monoclonal anti-human blood group B antibodies (HEB-29; Exbio, Praha, Czech) were used in this experiment. Phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), cardiolipin (CL), and $II^6NeuAc\alpha$-lactose were purchased from Sigma (St Louis, MO, USA). The concentrations of standard phospholipids in chloroform/methanol (1:1, v/v) were determined by the phosphomolybdate procedure after decomposition of the lipids with 70% $HClO_4$ and H_2O_2 (*14*).

Human blood

Normal human blood, blood groups A, B, AB and O, was obtained from Keio University Hospital, and used according to the guidelines of the Committee for Informed Consent. Blood was separated into erythrocytes and plasma by centrifugation at 2,000 rpm for 10 min. Erythrocytes were haemolysed with 0.1% acetic acid in water and then centrifuged at 6,000*g* for 20 min to obtain the membrane fraction, which was washed with water until haemoglobin had been reduced sufficiently. While the plasma was left at 4°C for 6 h and the fibrin produced was removed to obtain serum.

Quantitation of bacterial lipids

The lipid extracts from bacteria were partitioned by Folch's procedure (*15*), and then, aliquots corresponding to 0.5–1.0 mg dry weight, were separated on glass-coated (Merck, Darmstadt, Germany) and plastic-coated (Macherey-Nagel, Düren, Germany) TLC plates with chloroform/methanol/water (65:25:4. v/v/v). Detection was performed with cupric acetate–phosphoric acid reagent for all lipids, Dittmer's reagent for phospholipids, ninhydrin reagent for aminolipids and orcinol-sulphuric acid reagent for glycolipids, and by TLC-immunostaining with rabbit anti-LJ antisera (1:500), murine anti-B antibodies (1:500) and human sera (1:100).

Enzyme-linked immunosorbent assay

A purified glycolipid from LJ was dissolved in ethanol by sonication (0.5 μg/100 μl) and then put in each well of a microtitre plate, which was left at room temperature until the ethanol had completely evaporated off. The plate was blocked with 100 μl of bovine serum albumin (BSA) in PBS (1%) at 4°C overnight, and then to the plate, 100 μl of human serum diluted 1:100 and 1:200 with 1% BSA in PBS was added, followed by reaction at room temperature for 2 h. After washing the plate with 0.1% Tween-20 in PBS 5×, the antibodies bound on the plate were detected by reaction with 100 μl of peroxidase-conjugated anti-human IgG, A and M antibodies diluted 1:2,000 with 1% BSA in PBS at room temperature for 2 h, followed by reaction with *o*-phenylenediamine (4.6 mM) and H_2O_2 (0.015%) in 25 mM citrate–phosphate buffer (pH 5.0) as the substrates for peroxidase at room temperature for 15 min. The reaction was terminated by the addition of 4 M sulphuric acid (20 μl), and then the optical density at 490 nm was determined. The background values obtained on reaction with human sera in wells without glycolipids under the same conditions, were subtracted from the values obtained above.

Purification of glycolipids

The neutral and acidic lipid fractions by DEAE-Sephadex (A-25, acetate form; GE Healthcare Bioscience, Piscatway, NJ, USA) column chromatography contained glycolipids, and CL and PG, respectively, and after Folch's partitioning, they were purified using a silica gel (Iatrobeads 6RS8060: Iatron Laboratory, Tokyo) column by gradient elution with chloroform/methanol/water (85:15:0.2, 70:30:4 and 10:90:4, v/v/v). The purity of purified lipids was examined by TLC as described above.

Structural analysis of glycolipids

The purified glycolipids were analysed by positive ion FAB–MS (JMS-700TKM; JEOL, Tokyo) with triethanolamine (TEA) as the matrix and proton magnetic resonance spectroscopy (JNM-ECP700, JEOL) with dimethyl sulphoxide-d6/D_2O (98:2, v/v). For determination of the compositions of fatty acids and carbohydrates, they were methanolized with 5% HCl in methanol at 80°C for 16 h. The resulting fatty acid methyl esters were extracted with *n*-hexane, and 1-*O*-methyl hexoses in the methanol phase were converted to trimethylsilyl derivatives with pyridine/hexamethyl disilazane/trimethylchlorosilane (10:2:1, v/v/v) at 60°C for 5 min, followed by analysis with a GC–MS (GP5050: Shimadzu, Kyoto) equipped with a DB-1 column (0.25 mmø × 30 m) from 150°C to 250°C at 10°C/min. Also, linkage analysis of carbohydrates was carried out by conversion of glycolipids to partially methylated aldohexitol acetates, followed by analysis with a GC–MS with a DB-1 column from 150°C to 210°C at 4°C/min (*16*). Standard partially methylated

aldohexitol acetates were obtained from the following sources: terminal Glc from GlcCer, 2-linked Glc from kojibiosyl glycerol, 3-linked Glc from nigerosy glycerol, 4-linked Glc from LacCer and Gb_4Cer, 6-linked Glc from 6-glucosyl dextran, terminal Gal from GalCer, 3- and 4-linked Gal from Gb_4Cer, 2-linked Gal from $IV^2Fuc\text{-}Gg_4Cer$, and 6-linked Gal from II^6NeuAc lactose. In addition, for determination of the carbohydrate sequences and anomeric configurations, glycolipids were hydrolysed with the following glycosidases: α-glucosidase (rice: Sigma) in 50 mM citrate buffer (pH 4.5), β-glucosidase (almond: Wako, Tokyo) in 50 mM citrate buffer (pH 6.5), and α-galactosidase (coffee bean: Sigma) in 50 mM citrate buffer (pH 4.5). Glycolipids (~2 μg) together with 100 μg/μl sodium taurocholate were hydrolysed by incubation with the enzyme at 37°C for 1–16 h in a final volume of 100 μl, and the products were recovered by solvent partitioning with 100 μl of chloroform and 50 μl methanol, followed by with 100 μl of methanol/water (1:1, v/v), and were examined by TLC with chloroform/methanol/water (65:25:4, v/v/v) and orcinol-sulphuric acid reagent for glycolipids, and with *n*-hexane/diethyl ether/acetic acid (80:30:4, v/v/v) and cupric acetate–phosphoric acid reagent for diacylglycerol.

Results

Bacterial lipids

As shown in Fig. 1, the total lipid extracts from several bacteria were examined by TLC and TLC-immunostaining. In accord with previous reports (*17*, *18*), the amounts of PE (4.8 ± 0.8 μg/mg dry weight) and CL plus PG (3.2 ± 0.7 μg/mg dry weight) in *E. coli*, as a Gram-negative bacterium, were exceedingly higher than those in the other Gram-positive bacteria, and glycolipids were absent in *E. coli*. This was the reason why glycolipids in Gram-negative bacteria have been characterized as lipooligosaccharides with lipid A as the hydrophobic moiety in the outer membrane of the cell wall, and why they can not be extracted with chloroform/methanol (*19*). In contrast, the major phospholipids in Gram-positive bacteria, i.e. *Lactobacillus*, *Staphylococcus* and *Streptococcus* species, were CL and PG, and DH-DG was present in significantly high amounts (0.34–2.05 μg/mg dry weights), indicating that the cytoplasmic membranes of Gram-positive bacteria are mainly composed of glycerophospholipids and glyceroglycolipids.

Although PG and DH-DG were present in all Lactobacilli examined, CL, TH-DG and TetH-DG were present in relatively high concentrations in the following strains: CL in LJ, *L. reuteri, L. fermentum* and *L. plantarum,* and TH-DG in LJ, *L. intestinalis* (LI), *L.casei* and *L. rhamnosus,* and TetH-DG in LJ and LI (Table I). On TLC-immunostaining with anti-LJ antisera, DH-DG, TH-DG and TetH-DG in *Lactobacillus* species exhibited a positive reaction, and TH-DG and TetH-DG were detected with sensitivity of more than 10 times higher than that in the case of DH-DG, but DH-DG in *Staphylococcus* and *Streptococcus* species were not reactive with the antisera at all (Fig. 1). DH-DG was confirmed to be a common antigenic glycolipid in *Lactobacillus* species, and TH-DG and TetH-DG were revealed to exhibit a restricted distribution in *Lactobacillus* species.

Glycolipids in LJ

The major glycolipids, DH-DG, TH-DG and TetH-DG, in LJ were purified by means of DEAE-Sephadex and Iatrobeads column chromatographies

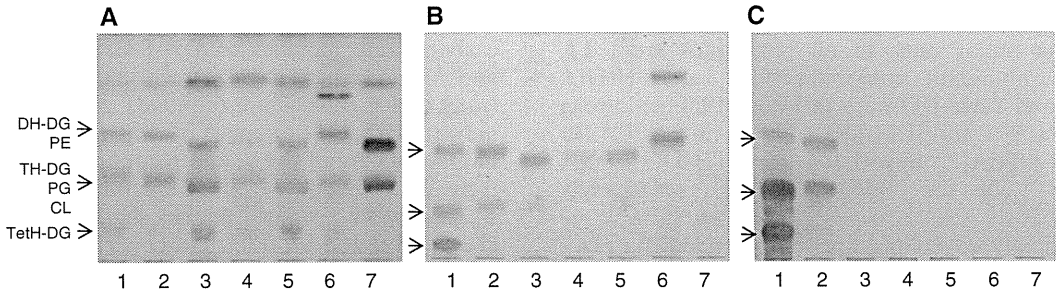

Fig. 1 TLC and TLC-immunostaining of lipids from several bacteria. Lipids, corresponding to 0.5 mg dry weight, were developed on glass-coated (A and B) and plastic-coated (C) TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were detected with cupric acetate–phosphoric acid (A), orcinol-sulphuric acid (B) and anti-LJ antisera (1:500 dilution) (C). 1, LJ; 2, *L. casei*; 3, *S. aureus* (ATCC 860); 4, *S. epidermidis* (JCM2414); 5, *S. epidermidis* (ATCC866); 6, *S. salivalis*; 7, *E. coli*. Arrows beside each TLC plate indicate the positions of DH-DG, TH-DG and TetH-DG, from the top, respectively.

Table I. Amounts of lipids in several lactobacilli. (μg/mg dry weight).

	Lactobacillus johnsonii	*Lactobacillus intestinalis*	*Lactobacillus reuteri*	*Lactobacillus casei*	*Lactobacillus fermentum*	*Lactobacillus plantarum*	*Lactobacillus rhamnosus*
Phospholipids							
CL	1.14	0.18	1.11	0.10	1.45	0.35	0.05
PG	0.69	0.11	0.58	1.91	0.54	0.30	0.72
Glycolipids							
MH-DG	0.15						
DH-DG	1.36	0.22	0.99	2.22	1.54	0.93	0.81
TH-DG	1.10	0.17	0.31	0.96			0.63
TetH-DG	0.93	0.15	tr				

tr, trace amount, <0.01 μg/mg dry weight.

with monitoring of their reactivities with orcinol-sulphuric acid and anti-LJ antisera (lanes 1, 2 and 3 in Fig. 2). Their carbohydrate compositions, as determined by GC–MS of 1-*O*-methyl 2,3,4,6-tetraTMS derivatives, indicated that DH-DG, TH-DG and TetH-DG in LJ contained Glc and Gal in the ratios of 1:1, 1:2 and 1:3, respectively (Table II). Hydrolysis of them with α-galactosidase (coffee bean) yielded MH-DG, whose carbohydrate was proven to be Glc by GC–MS, and which was further converted to DG by subsequent treatment with α-glucosidase (rice) (Fig. 3). These results indicated that both Glc and Gal were of the α-configurations, and α-Gal was sequentially linked with Glc-DG in DH-DG, TH-DG and TetH-DG. Then, linkage analysis of the carbohydrate moieties was performed by GC–MS of partially methylated aldohexitol acetates prepared from them. As shown in Fig. 4, partially methylated aldohexitol acetates of TetH-DG from LJ gave three peaks, whose retention times and mass spectra were identical with those of 1,5-di-*O*-acetyl, 2,3,4,6-tetra-*O*-methyl galactitol (terminal Gal) for peak a, 1,2,5-tri-*O*-acetyl 3,4,6-tri-*O*-methylglucitol (2-linked Glc) for peak b, and 1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methyl galactitol (6-linked Gal) for peak c, and their peak ratio compared to that of 2-linked Glc was 1:1:2, respectively (Table II). Thus, the structure of TetH-DG from LJ was concluded to be Galα1-6Galα1-6Galα1-2Glcα1-3′DG. In a similar way, TH-DG and DH-DG from LJ were determined to be Galα1-6Galα1-2Glcα1-3′DG and Galα1-2Glcα1-3′DG, respectively (Table II and Fig. 3). Concerning the fatty acid compositions (Table III), 11-octadecenoic acid (18:1, cis-baccenic acid) and 11,12-methylene-octadecanoic acid (cyclopropane-19:0, c19:0, lactobacillic acid) were found to be the major ones in all glycolipids from LJ, and the abundant molecular ions of DH-DG, TH-DG and TetH-DG were 18:1 and c19:0-containing ones, m/z 982 for $[DH\text{-}DG + Na]^+$, m/z 1,108 for $[DH\text{-}DG + TEA]^+$, m/z 1,144 for $[TH\text{-}DG + Na]^+$ and m/z 1,306 for $[TetH\text{-}DG + Na]^+$, respectively (Fig. 5).

Accordingly, glycolipid antigens detected on TLC-immunostaining with anti-LJ antisera were found to carry a common α-Gal residue as the antigenic determinant at the non-reducing terminals of glycolipids, because no reaction of antisera with Glcα1-3′DG (MH-DG) was observed. Also, the binding capacities of antibodies as to TH-DG and TetH-DG were significantly higher than that as to DH-DG, and >2 ng of TH-DG and TetH-DG, corresponding to 1×10^6 cells of LJ, could be detected on TLC-immunostaining (Fig. 2) (*2*).

Glycolipids in other bacteria

The major glycolipids in Fig. 1 were also purified as described above, and their structures were elucidated by permethylation analysis and exoglycosidase treatment (Table II). DH-DG, TH-DG and TetH-DG from LI were identical with those from LJ, and DH-DGs from *L. reuteri, L. fermentum, L. plantarum, L. rhamnosus* and *L. casei* were also proven to be Galα1-2Glcα1-3′DG as a common glycolipid in *Lactobacillus* species (Table II). However, DH-DG from *L. casei* was separated into two bands, one exhibiting identical mobility with that of DH-DG from LJ and the other migrating above DH-DG, it being slightly less polar than DH-DG. The less polar glycolipid was demonstrated to be an esterified derivative of TH-DG with 16:0, 18:0 and c19:0 as the fatty acids and with terminal non-esterified hexose by positive ion FABMS (data not shown). As shown in Table II, permethylation analyses of TH-DG and esterified TH-DG revealed that they were Glc1-6Gal1-2Glc1-3′DG and Glc1-6Gal1-2Glc(6-fatty acid)1-3′DG, respectively, which were identical with those reported in the literature (*10*, *11*).

On the other hand, glucose was the sole carbohydrate in the DH-DGs from *S. epidermidis* and *S. salivalis*, whose oligosaccharide structures were shown to be Glc1-6Glc1-3′DG and Glc1-2Glc1-3′DG, respectively, by permethylation analysis (Table II). The carbohydrate moieties of DH-DGs from *S. salivalis* and *S. epidermidis* were susceptible to α-glucosidase (rice) and β-glucosidase (almond), DG being yielded, indicating that the anomers of the two glucose residues in DH-DG are of the α- and β-configurations, respectively. In addition, ^1H NMR spectra of DH-DG from *S. epidermidis* contained doublet signals at $\delta = 4.26$ ppm ($J = 7.8$ Hz) and $\delta = 4.02$ ppm ($J = 7.7$ Hz) due to gentibiose. Thus, in accord with previous reports, the major glycolipids from *S. epidermidis* and *S. salivalis* were Glcβ1-6Glcβ1-3′DG, gentibiosyl DG, and Glcα1-2Glcα1-3′DG, kojibiosyl DG, respectively (*5*, *7*).

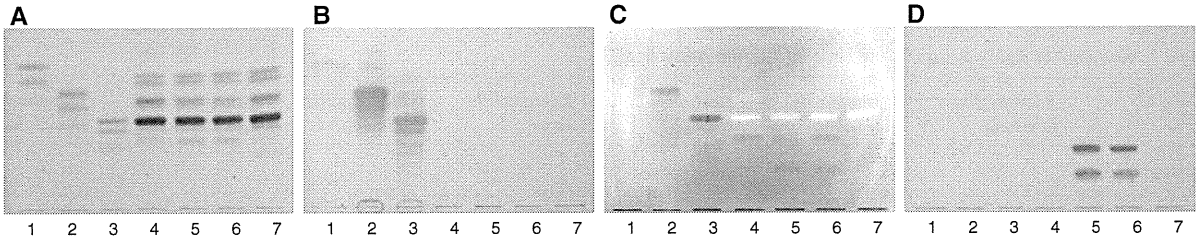

Fig. 2 TLC and TLC-immunostaining of purified glycolipids from LJ and neutral glycolipids from human erythrocytes. DH-DG (*1*), TH-DG (*2*) and TetH-DG (*3*) purified from LJ, 0.5 μg of each, and neutral glycolipids from human erythrocyte membranes, blood groups A (*4*), B (*5*), AB (*6*) and O (*7*), corresponding to 0.5 mg of dried membranes, were developed on plastic-coated TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were detected with orcinol-sulphuric acid (A) and anti-LJ antisera (1:500 dilution) (B), human blood group O serum (1:100 dilution)(C) and anti-blood group B antibodies (1:500 dilution) (D). The two bands of DH-DG, TH-DG and TetH-DG, respectively, represent molecular species with different fatty acids.

Table II. Carbohydrate compositions determined by GC–MS of TMS derivatives of carbohydrates and partially methylated aldohexitol acetates of glycolipids from several bacteria.

	TMS derivatives		Partially methylated aldohexitol acetates					
	Glc	Gal	t-Glc	t-Gal	2-Glc	6-Glc	2,6-Glc	6-Gal
Lactobacillus johnsonii								
DH-DG	1	0.9	nd	1.1	1	nd	nd	nd
TH-DG	1	1.9	nd	1.2	1	nd	nd	1.1
TetH-DG	1	2.8	nd	0.9	1	nd	nd	2.2
Lactobacillus intestinalis								
DH-DG	1	1.1	nd	1.1	1	nd	nd	nd
TH-DG	1	1.8	nd	0.9	1	nd	nd	1.1
TetH-DG	1	2.8	nd	0.9	1	nd	nd	2.0
Lactobacillus casei								
DH-DG	1	1.2	nd	1.2	1	nd	nd	nd
TH-DG	1	0.5	1.1	nd	1	nd	nd	1.2
Esterified TH-DG	1	0.5	1.1	nd	nd	nd	1	1.2
Staphylococcus epidermidis								
DH-DG	1	nd	1	nd	nd	0.8	nd	nd
Streptococcus salivalis								
DH-DG	1	nd	1	nd	1.1	nd	nd	nd

Peak areas of TMS-Gal were compared to that of TMS-Glc. Peak areas of partially methylated aldohexitol acetates were compared to those of 2-linked Glc for *Lactobacillus* glycolipids, 2,6-linked Glc for esterified TH-DG from *L. casei,* and terminal Glc for glycolipids from *S. epidermidis* and *S. salivalis.* Terminal Gal and 2-linked Glc in DH-DGs from *L.reuteri, L.fermentum, L.plantarum and L.rhamnosus* were also detected in the ratio of 1:1. t-Glc, terminal Glc (1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylglucitol); t-Gal, terminal Gal (1,5-di-*O*-acetyl 2,3,4,6-tetra-*O*-methylgalactitol); 2-Glc, 2-linked Glc (1,2,5-tri-*O*-acetyl 3,4,6-tri-*O*-methylglucitol); 6-Glc, 6-linked Glc (1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methylglucitol); 2,6-Glc, 2,6-linked Glc (1,2,5,6-tetra-*O*-acetyl 3,4-di-*O*-methylglucitol); 6-Gal, 6-linked Gal (1,5,6-tri-*O*-acetyl 2,3,4-tri-*O*-methylgalactitol); nd, not detected.

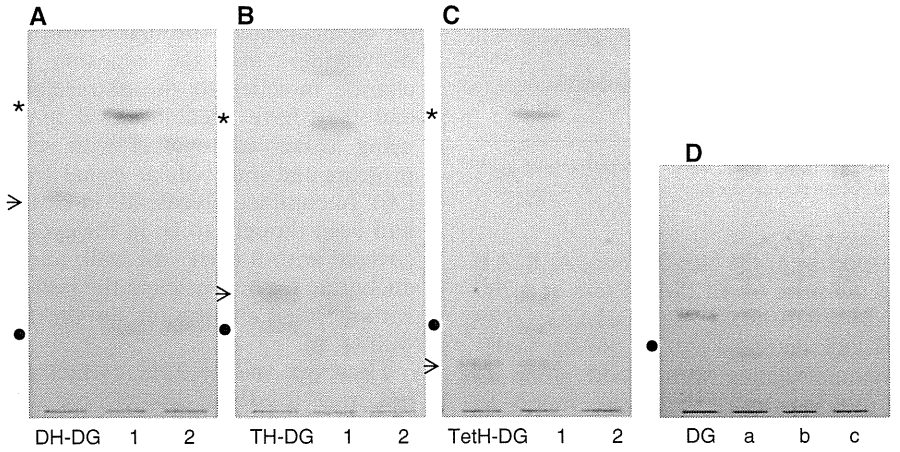

Fig. 3 TLC of the products on treatment with α-galactosidase and α-glucosidase. The products on hydrolysis of DH-DG (A), TH-DG (B) and TetH-DG (C) by α-galactosidase (coffee bean) (*1*), followed by α-glucosidase (rice) (*2*), as described in the text, were developed on TLC plates with chloroform/methanol/water (65:25:4, v/v/v), and were visualized with orcinol-sulphuric acid reagent. The products of DH-DG (a), TH-DG (b) and TetH-DG (c) on hydrolysis with α-galactosidase and α-glucosidase were also developed with *n*-hexane/diethyl ether/acetic acid (80:30:4, v/v/v), and were visualized with cupric acetate–phosphoric acid reagent (D). Arrows besides plates A–C indicate the positions of DH-DG, TH-DG and TetH-DG, respectively. Asterisk, MH-DG; filled circle, sodium taurocholate-derived spots.

The structures determined of glycolipids from several bacteria in this study are summarized in Table IV.

On the other hand, the fatty acid compositions of DH-DG from several bacteria were also found to be bacterial species-characteristic, c19:0 being preferentially present in those from *Lactobacillus* species, and anteiso15:0 and anteiso17:0 in DH-DG from *S. epidermidis* (Table III).

Antibodies toward glycolipids from LJ in human sera

Because DH-DG, TH-DG and TetH-DG from LJ exhibited strong antigenicities, antibodies toward them in human sera were examined by enzyme-linked immunosorbent assay (ELISA) and TLC-immunostaining. On ELISA, all human sera (36 cases) were revealed to contain antibodies toward them, with various titres, and the antibody titer as to TetH-DG in individual sera was constantly higher than those as to DH-DG

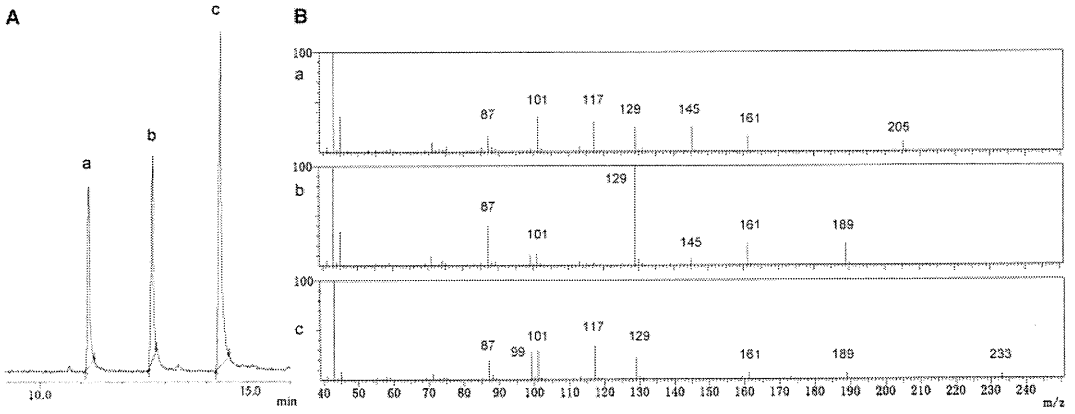

Fig. 4 GC–MS chromatogram (A) and mass spectra (B) of partially methylated aldohexitol acetates prepared from TetH-DG of LJ. Partially methylated aldohexitol acetates were prepared from TetH-DG of LJ and were analysed by GC–MS. Mass spectra a–c correspond to peaks a–c, which were characterized as terminal Gal, 2-linked Glc and 6-linked Gal, respectively.

Table III. Fatty acid compositions of DH-DG from several bacteria.

	Lactobacillus johnsonii	*Lactobacillus casei*	*Staphylococcus epidermidis*	*Streptococcus salivalis*
14:0	0.5	–	2.6	10.9
14:1	–	–	0.8	–
15:0	–	–	–	–
a15:0	–	–	66.8	–
16:0	2.2	15.5	5.1	50.6
16:1	–	–	1.1	9.7
a17:0	–	–	19.4	–
18:0	–	7.5	4.2	5.4
18:1	57.7	36.6	–	23.4
18h:0	10.1	7.8	–	–
c19:0	29.5	32.6	–	–

a, anteiso; c, cyclopropane; h, hydroxy; 18:1, 11-octadecenoic acid; c19:0, 11,12-methylene-octadecanoic acid.

and TH-DG, irrespective of the blood group, A, B, AB or O. The optical densities on ELISA of sera at 1:200 dilution were 0.25 (0.01–0.68) for DH-DG, 0.39 (0.1–1.14) for TH-DG, and 0.64 (0.08–1.96) for TetH-DG. On TLC-immunostaining with blood group O serum at 1:100 dilution, antibodies were found to bind with TH-DG, TetH-DG, and blood group A- and B-glycolipids, and the density of TetH-DG was three times higher than that of TH-DG (Fig. 2C). Since rabbit anti-LJ antiserum reacted with DH-DG, TH-DG and TetH-DG, but not with B-glycolipids (Fig. 2B), and Gb_3Cer and $IV^3Gal\alpha\text{-}nLc_4Cer$ (*1*), and reversely anti-blood B antibodies reacted with B-glycolipids, but not all glycolipids from LJ (Fig. 2D), the α-Gal residues in glycolipids from LJ seemed not to cross-react with that of B-glycolipids containing α-Gal (Fig. 2). Similar to in the cases of A and B-glycolipids, TetH-DG after reaction with human sera more intensively stained with peroxidase-conjugated anti-human IgM antibodies than with anti-human IgA and anti-human IgG ones, indicating that human antibodies against TetH-DG are mainly of the IgM isotype. Thus, an immune reaction against antigenic glycolipids in lactobacilli was shown to occur in the human body, probably as a result of bacterial symbiosis.

Discussion

As reported in this article, glycolipids in both bacteria and the hosts were revealed to play a role in mutual recognition. That is, glycolipids in LJ provided the antigenic determinant for the immune recognition of host animals, and those in the tissues and cells of animals provided the receptor for bacterial attachment. In general, molecular functions of glycolipids are the anchoring of functional carbohydrate chains on biomembranes through hydrophobic ceramides or diglycerides, and the exposure of their oligosaccharide moieties to the extracellular circumstances, and they have been localized in the raft structure, being assembled together with several physiological proteins and intracellular signal transduction systems (*20*). In contrast, glycolipids in Gram-positive bacteria had been thought to be in the cytoplasmic membrane covered with a thick cell wall, and to play a role by anchoring teichoic acids to the wall as lipoteichoic acid without their expression on the surface of the cell wall (*4–6*).

However, in the lipid extract from LJ, we were not able to detect lipoteichoic acid, probably due to the different extraction and structural characterization procedures from those described in the literature (*4–6*), or due to removal of hydrophilic materials from the lipid extract on Folch's partitioning (*15*). As shown in Fig. 1, the major lipids in the Folch's lower phase from LJ were phospholipids and glycolipids. In our previous study (*1*), we determined the amounts of the major phospholipids, CL and PG, by TLC-densitometry after visualization with cupric acetate–phosphoric acid, but the densities of CL and PG from LJ were found to be significantly lower than those of standard CL and PG in the same molar amounts, probably due to the difference in their fatty acid compositions. Accordingly, in this study, we used Dittmer's reagent for determination of CL and PG by TLC-densitometry, for which the plates, after spraying of the reagent, were kept at 4°C for more than 4 h to reduce the background. In the Folch's lower phase, the amounts of glycolipids,

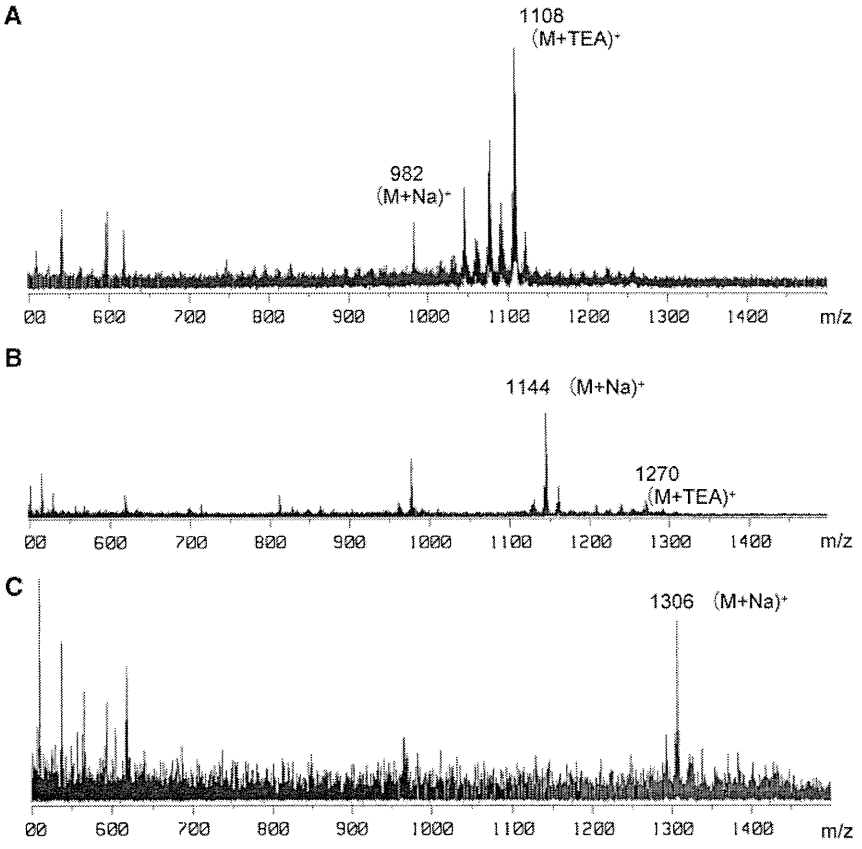

Fig. 5 Positive ion FABMS spectra of DH-DG (A), TH-DG (B) and TetH-DG (C) from LJ. Purified glycolipids (5 μg) in 5 μl C/M (1:1, v/v) were mixed with TEA (~5 μl) on a target plate and then analysed by FABMS.

Table IV. Structures of glycolipids from several bacteria.

Bacteria	Glycolipids	Structures
Lactobacillus johnsonii	DH-DG	Galα1-2Glcα1-3′DG
Lactobacillus intestinalis	TH-DG	Galα1-6Galα1-2Glcα1-3′DG
	TetH-DG	Galα1-6Galα1-6Galα1-2Glcα1-3′DG
Lactobacillus reuteri	DH-DG	Galα1-2Glcα1-3′DG
Lactobacillus fermentum		
Lactobacillus plantarum		
Lactobacillus rhamnosus		
Lactobacillus casei	DH-DG	Galα1-2Glcα1-3′DG
	TH-DG	Glcl-6Galα1-2Glcα1-3′DG
		Glcl-6Galα1-2Glc(6-fatty acid)α1-3′DG
Staphylococcus epidermidis	DH-DG	Glcβ1-6Glcβ1-3′DG
Streptococcus salivalis	DH-DG	Glcα1-2Glcα1-3′DG

DH-DG, TH-DG and TetH-DG, were equivalent to those of phospholipids, indicating that the majority of glycolipids are present without teichoic acids as the membrane constituents in LJ. Also, since anti-glycolipid antisera have been shown to react with the respective bacteria, and can be used as probes for bacterial classification (*9*, *10*), glycolipids seemed to be partly expressed on the surface of the cell wall. In fact, bacterial agglutination with anti-LJ antisera was inhibited by half of the activity after absorption of antisera with TetH-DG-containing liposomes according to the procedure reported previously (data not shown) (*21*).

A common structure of glycolipid antigens in LJ is α-Gal at their non-reducing terminals, and the extended structures in DH-DG, TH-DG and TetH-DG exhibited strong antigenicities in that order. To a lesser extent, TH-DH and esterified TH-DG, whose non-reducing terminals are Glc, in *L. casei* also reacted, but Glcα1-2Glcα1-3′DG in *S. salivalis* and Glcβ1-6Glcβ1-3′DG in *S. epidermidis* did not react with anti-LJ antisera at all, indicating that Galα1-2Glcα is a minimal structure for recognition by anti-LJ antisera and its modification with Glc at the non-reducing terminal does not significantly affect the reactivity. The non-reducing terminal Gal extended by an α1-6

linkage in TH-DG and TetH-DG from LJ was found to enhance the antigenicity of DH-DG, and therefore the Galα1-6 group is an antigenic determinant for LJ.

As to the glycolipid composition in LI, the relative amounts of TH-DG and TetH-DG were significantly higher than those reported previously (Fig. 2C) (*1*). Although the glycolipid composition of the original LI was 92% DH-DG, 6% TH-DG and <2% TetH-DG, immunization of rabbits with the original LI generated antibodies that reacted strongly with TetH-DG in LJ as well as with TetH-DG in LI, indicating that the original LI has TetH-DG. On structural characterization, DH-DG and TH-DG from the original LI, and DH-DG, TH-DG and TetH-DG from LI in this study were proven to have the same structures as those from LJ. Continuous subculture of LI for more than 1 year seemed to result in a change in glycolipids, 40.7% DH-DG, 31.5% TH-DG and 27.8% TetH-DG, but the change in the lipid compositions in other bacteria on continuous subculture was estimated to be <10%. To clarify the genetic background of the differences in glycolipid composition between the original and long-term cultured LI, and among various bacteria, analysis of sugar transferase genes is now in progress in our laboratory. In conclusion, TetH-DG was detected in intestinal lactobacilli, i.e. LJ, LI, *L. reuteri* and LJ isolated from the cecal and colonal contents of mice (*2*).

The strong antigenicity of TetH-DG was revealed not only on characterization of antigens for rabbit anti-LJ antisera, but also from its reactivity with normal human sera. As described above, antibodies toward TH-DG and TetH-DG were readily generated on immunization of rabbits with LJ and LI, even with <0.01 μg/mg dry weight of the original LI, and they were frequently detected in normal human sera as a result of an immune reaction against them, probably for protection from bacterial invasion of human body. In Peyer's patches, bacteria bound with receptors were shown to be incorporated into the M cells in the follicle-associated epithelium and to be transported to gut-associated lymphoid tissues, where secretory IgA was produced and secreted into the mucosal layer of the digestive tract to prevent bacterial invasion and irregular diffusion of bacteria in the mucosal layer (*22*, *23*). Also, production of IgM as an event in the initial immune reaction was also supposed to occur for protection from bacterial invasion of the blood circulation system, because IgM is the major immunoglobulin with strong agglutination activity in human sera. Since anti-LJ antisera did not cross-react with α-Gal containing sphingoglycolipids, i.e. Gb_3Cer, $IV^3Gal\alpha$-nLc_4Cer and blood group B-antigen, antibodies toward TH-DG and TetH-DG in symbiotic lactobacilli seemed not to react with the tissues or cells of the host animal (*1*).

On the other hand, although the Gg_4Cer-like structure was detected in the protein fraction of LJ on Western blotting and antibodies against it were generated on immunization of rabbits with LJ (*1*, *2*), it was not detected in normal human sera even on dilution to 1:32. While, on infection with *Campylobacter jejuni* cells containing oligosaccharides mimicking Gg_4Cer and gangliosides, antibodies against them have been revealed to be produced occasionally and to be closely associated with the onset of autoimmune diseases such as Guilain–Barré syndrome (*24*, *25*). Probably, the whole oligosaccharides profile in individual bacteria is important for immunocytes to distinguish bacterial species, even if Gg_4Cer is a strong antigenic molecule. Accordingly, one can suggest that oligosaccharide profiles recognized by antibodies in normal human sera reveal the bacterial species with symbiosis or infection.

Since several bacteria, i.e. LJ, *L. reuteri, L. casei, Bifidobacterium bifidum, Pseudomonous aeruginosa, Actinomyces maeslundii* and *Neiseria gonorrhoeae,* utilize Gg_4Cer as a receptor, suppression of competitive or injurious bacteria through the structural modification of Gg_4Cer seems to be important for establishment of symbiosis or protection from infection, respectively. Gg_4Cer in the murine digestive tract is known to be structurally modified in a region-characteristic mode. Namely, Gg_4Cer in the stomach, cecum and colon is completely converted to its fucosylated derivative by α1,2-fucosyltransferase from the FUT-2 gene product, but >85% was expressed in the small intestine of conventionally breeding mice (*2*, *26*). The modification of Gg_4Cer to $IV^2Fuc\alpha$-Gg_4Cer has not been observed in germ-free mice, but has been revealed to occur on transcriptional induction of the FUT-2 gene on infection by indigenous filamentous bacteria and wild-type *Bacteroides thethaiotaomicron*, indicating that the expression of the FUT-2 gene is regulated epigenetically (*27*, *28*). Also, in FUT-2 gene null mice, although $IV^2Fuc\alpha$-Gg_4Cer is completely absent in the stomach, cecum and colon, its synthesis in the small intestine is maintained through alternative expression of the FUT-1 gene, indicating that modification of Gg_4Cer by fucosyltransferase in the small intestine is maintained in both FUT-1 and FUT-2 null mice, and is indispensable for regulation of the receptor activity of Gg_4Cer under conventionally breeding conditions in both FUT-1 and FUT-2 null mice (*26*). So far, it is unclear whether the epigenetic expression of the fucosyltransferase gene in the small intestine is due to bacteria or the immune response of the host.

In addition, Gg_4Cer, corresponding to ~20% of the total amount in the whole digestive tract, was present in the digestive tract contents and excreted into the faeces without degradation. The amounts of Gg_4Cer and LJ in the caecal and colonic contents were 37 μg versus 9.8×10^7 cells and 49 μg versus 1.4×10^8 cells, respectively (*2*). Consequently, Gg_4Cer in the caecal contents corresponded to 1.6×10^{16} molecules, which is sufficient for one-to-one binding of 9.8×10^7 cells of LJ, indicating that Gg_4Cer in the contents might facilitate the discharge of intestinal bacteria by becoming attached to them to prevent their irregular diffusion (*2*). Thus, glycolipids in the tissues of host animals were revealed to be actively metabolized, and to participate in the attachment of bacteria to the receptors and the discharge of bacteria from the body.

Conflict of interest
None declared.

References

1. Iwamori, M., Shibagaki, T., Nakata, Y., Adachi, S., and Nomura, T. (2009) Distribution of receptor glycolipids for Lactobacilli in murine digestive tract and production of antibodies cross-reactive with them by immunization of rabbits with Lactobacilli. *J. Biochem.* **146**, 185–191
2. Iwamori, M., Iwamori, Y., Adachi, S., and Nomura, T. (2011) Excretion into feces of asialo GM1 in the murine digestive tract and Lactobacillus johnsonii exhibiting binding ability toward asialo GM1. A possible role of epithelial glycolipids in the discharge of intestinal bacteria. *Glycoconj. J.* **28**, 21–30
3. Kojima, N., Uchikawa, K., Araki, Y., and Ito, E. (1985) A common linkage saccharide unit between teichoic acids and peptidoglycan in cell walls of Bacillus coagulans. *J. Biochem.* **97**, 1085–1092
4. Fischer, W., Schuster, D., and Laine, R.A. (1979) Studies on the relationship between glycerophosphoglycolipids and lipoteichoic acids. IV. Trigalactosylglycerophospho-acylkojibiosyldiacylglycerol and related compounds from *Streptococcus lactis Kiel* 42172. *Biochim. Biophys. Acta* **575**, 389–398
5. Shaw, N. and Baddiley, J. (1968) Structure and distribution of glycosyl diglycerides in bacteria. *Nature* **217**, 142–144
6. Koch, H.U. and Fischer, W. (1978) Acyldiglucosyldiacylglycerol-containing lipoteichoic acid with a poly(3-O-galabiosyl-2-O-galactosyl-sn-glycero-1-phosphate) chain from *Streptococcus lactis Kiel* 42172. *Biochemistry* **17**, 5275–5281
7. Shaw, N. (1970) Bacterial glycolipids. *Bacteriol. Rev.* **34**, 365–377
8. Smith, P.F. (1972) Lipid composition of *Mycoplasma neurolyticum*. *J. Bacteriol.* **112**, 554–558
9. Sugiyama, T., Smith, P.F., Langworthy, T.A., and Mayberry, W.R. (1974) Immunological analysis of glycolipids and lipopolysaccharides derived from various mycoplasmas. *Infect. Immun.* **10**, 1273–1279
10. Hirsch, H.E. and Parks, M.E. (1976) Serological reactions against glycolipid-sensitised liposomes in multiple sclerosis. *Nature* **264**, 785–787
11. Nakano, M. and Fischer, W. (1978) Trihexosyldiacylglycerol and acyltrihexosyldiacylglycerol as lipid anchors of the lipoteichoic acid of *Lactobacillus casei* DSM 20021. *Hoppe Seylers Z. Physiol. Chem.* **359**, 1–11
12. Ntamere, A.S., Taron, D.J., and Neuhaus, F.C. (1987) Assembly of D-alanyl-lipoteichoic acid in *Lactobacillus casei*: mutants deficient in the D-alanyl ester content of this amphiphile. *J. Bacteriol.* **169**, 1702–1711
13. IUPAC-IUB Commission on Biochemical Nomenclature. (1977) The Nomenclature of Lipids. *Eur. J. Biochem.* **179**, 11–21
14. Bartlett, G.R. (1959) Phosphorus assay in column chromatography. *J. Biol. Chem.* **234**, 466–468
15. Folch, J., Lees, M., and Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497–509
16. Iwamori, M., Takamizawa, K., Momoeda, M., Iwamori, Y., and Taketani, Y. (2008) Gangliosides in human, cow and goat milk, and their abilities as to neutralization of cholera toxin and botulinum type A neurotoxin. *Glycoconj. J.* **25**, 675–683
17. Shibuya, I., Yamagoe, S., Miyazaki, C., Matsuzaki, H., and Ohta, A. (1985) Biosynthesis of novel acidic phospholipid analogs in *Escherichia coli*. *J. Bacteriol.* **161**, 473–477
18. Asai, Y., Katayose, Y., Hikita, C., Ohta, A., and Shibuya, I. (1989) Suppression of the lethal effect of acidic-phospholipid deficiency by defective formation of the major outer membrane lipoprotein in *Escherichia coli*. *J. Bacteriol.* **171**, 6867–6869
19. Dzieciatkowska, M., Brochu, D., van Belkum, A., Heikema, A.P., Yuki, N., Houliston, R.S., Richards, J.C., Gilbert, M., and Li, J. (2007) Mass spectrometric analysis of intact lipooligosaccharide: direct evidence for O-acetylated sialic acids and discovery of O-linked glycine expressed by *Campylobacter jejuni*. *Biochemistry* **46**, 14704–14714
20. Füllekrug, J. and Simons, K. (2004) Lipid rafts and apical membrane traffic. *Ann. NY Acad. Sci.* **1014**, 164–169
21. Iwamori, M., Murata, M., Toyoda, M., and Iwamori, Y. (2009) Contribution of glycolipids to species-specific antigens on erythrocytes of several animal species as to recognition of antigens with rabbit anti-glycolipids and anti-erythrocyte antisera. *Glycoconj. J.* **26**, 467–476
22. Bockman, D.E. and Cooper, M.D. (1973) Pinocytosis by epithelium associated with lymphoid follicles in the bursa of Fabricius, appendix, and Peyer's patches. An electron microscopic study. *Am. J. Anat.* **136**, 455–477
23. Owen, R.L. and Jones, A.L. (1974) Epithelial cell specialization within human Peyer's patches: an ultrastructural study of intestinal lymphoid follicles. *Gastroenterology* **66**, 189–203
24. Jacobs, B.C., Rothbarth, P.H., van der Meché, F.G., Herbrink, P., Schmitz, P.I., de Klerk, M.A., and van Doorn, P.A. (1998) The spectrum of antecedent infections in Guillain-Barré syndrome: a case-control study. *Neurology* **51**, 1110–1115
25. Godschalk, P.C., Kuijf, M.L., Li, J., St Michael, F., Ang, C.W., Jacobs, B.C., Karwaski, M.F., Brochu, D., Moterassed, A., Endtz, H.P., van Belkum, A., and Gilbert, M. (2007) Structural characterization of *Campylobacter jejuni* lipooligosaccharide outer cores associated with Guillain-Barre and Miller Fisher syndromes. *Infect. Immun.* **75**, 1245–1254
26. Iwamori, M. and Domino, S.E. (2004) Tissue-specific loss of fucosylated glycolipids in mice with targeted deletion of alpha(1,2)fucosyltransferase genes. *Biochem. J.* **380**, 75–81
27. Lin, B., Hayashi, Y., Saito, M., Sakakibara, Y., Yanagisawa, M., and Iwamori, M. (2000) GDP-fucose: beta-galactoside alpha1,2-fucosyltransferase, MFUT-II, and not MFUT-I or -III, is induced in a restricted region of the digestive tract of germ-free mice by host-microbe interactions and cycloheximide. *Biochim. Biophys. Acta* **1487**, 275–285
28. Bry, L., Falk, P.G., Midtvedt, T., and Gordon, J.I. (1996) A model of host-microbial interactions in an open mammalian ecosystem. *Science* **273**, 1380–1383

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ORIGINAL PAPER

CRABP1-reduced expression is associated with poorer prognosis in serous and clear cell ovarian adenocarcinoma

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Abstract
Purpose CRABP1 is a modulator of retinoic acid function. The aim of the present study was to investigate CRABP1 expression and its clinical significance in ovarian carcinoma.
Methods Expression of CRABP1 protein was investigated by immunohistochemical analysis in 100 ovarian carcinomas of various histological sub-types, including serous and clear cell adenocarcinomas. Relationship of CRABP1 expression to clinical features, including prognosis, was analyzed.
Results Reduced expression of CRABP1 protein was detected especially frequently in the serous and clear cell adenocarcinomas sub-types, 50% (20 of 40) and 38% (10 of 26) of cases, respectively. We found that in both serous and clear cell adenocarcinomas overall survival was significantly poorer in the cases with reduced CRABP1 expression compared to similar cases where expression was maintained, irrespective of the disease stage ($P = 0.0073$ and 0.049, respectively). Disease-free survival of the serous and clear cell adenocarcinoma cases with reduced CRABP1 expression was significantly poorer, compared to the cases whose CRABP1 expression was maintained ($P = 0.024$). Multivariate analysis showed that reduced expression of CRABP1 was a significantly important prognostic factor (adjusted hazard ratio: 8.189 (95% CI, 2.186–30.672, $P = 0.0019$)).
Conclusions The present study is the first to demonstrate that the reduced expression of CRABP1 has a potential as a prognostic marker for serous adenocarcinoma which is the most frequent histological ovarian tumor type and also for clear cell carcinoma that often exhibits chemo-resistance. Further study is necessary to clarify how CRABP1 protein expression was altered and how CRABP1 affects ovarian carcinoma cells.

Keywords CRABP1 · Ovarian carcinoma · Serous · Clear cell · Prognosis

Abbreviations

CRABP1	Cellular retinoic acid binding protein 1
RA	Retinoic acid

Introduction

Ovarian cancer is the ninth most common cancer in US women (not counting skin cancers, the fifth leading cause of cancer deaths after lung and bronchus, breast, colorectal, and pancreatic cancers) and causes more deaths than any other cancer of the female reproductive system. In 2005, the last year in which accurate statistics were compiled in

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